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BULLETIN

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PASTEUR INSTITUTE

OF

SOUTHERN INDIA.

SECTION I.—NEGRI BODIES.

1.—On the differences in the occurrence of Negri bodies as a result of the subcutaneous inoculation of rabic virus derived (*a*) from the central nervous system, (*b*) from the salivary glands.

Having noticed in the course of systematic microscopical examination of the brains of animals, which had succumbed to rabies as a result of the subcutaneous inoculation of material from street virus brains, that Negri bodies often could not be seen or were very small and scanty, we were struck by the difference between the results of this artificial infection and of the natural infection by means of saliva. In natural rabies the infective matter is transferred in the medium of the saliva, and Negri bodies are almost invariably found in abundance, and of large size, in the hippocampus major. In artificial rabies, when the infective agent is matter from the central nervous system of a naturally infected animal, Negri bodies are nearly as often absent as present, and when present are generally of small size.

Subcutaneous infection with brain matter can never occur in nature, and the fact that natural infection by means of saliva almost invariably gives rise to one kind of microscopic appearances, whereas artificial infection with brain matter frequently fails to produce similar appearances, strengthens the opinion we have previously given expression to (Bulletin No. 1, 1908, page 22), that the saliva contains the rabies organism in one phase of development while the central nervous system contains it in another phase.

Of 31 guinea pigs, which had succumbed from time to time in various experiments to subcutaneous inoculation with street virus from the central nervous system, 16 showed no Negri bodies at all and in 15 they were found, but were seldom more than 2μ in diameter. Sub-inoculations were made subdurally in rabbits from the brains of two of the sixteen guinea pigs which showed no Negri bodies, with the result that the bodies re-appeared in large numbers from 5μ to 8μ in diameter.

Of 11 monkeys, which similarly had received street virus brain subcutaneously, 4 showed no Negri bodies, 6 showed scanty bodies up to 2μ and 1 showed numerous bodies up to 8μ. The last animal had received a very large dose of virus and had an incubation of only 13 days.

To ascertain whether large Negri bodies invariably appear after the subcutaneous inoculation of an emulsion of pounded salivary glands, which is the nearest approach to a natural infection that can be effected, 3 series of guinea pigs were inoculated, starting in each series with the salivary glands removed from a guinea pig which had succumbed to subdural inoculation with brain matter from a street virus dog.

No attempt was made to differentiate between the salivary glands, but from each guinea pig both parotids and both sub-maxillaries were carefully dissected out, freed as far as possible from blood, connective tissue and afferent nerves and pounded up with a little 0·85 per cent. salt solution. Of the resulting emulsion 0·5 cc. to 2 cc. was

injected into the abdominal subcutaneous tissue of 3 or 4 other guinea pigs. A similar procedure was adopted to continue the series, whenever one of the guinea pigs developed rabies.

Series 1 failed after the fourth salivary gland passage,
Series 2 after the fourth passage, and
Series 3 after the fifth passage.

Each series, however, probably could have been kept going indefinitely if only a large enough number of sub-inoculations could have been made.

Nevertheless the results obtained seem to be sufficiently consistent to point the distinction between virus derived from the central nervous system on the one hand and from the salivary glands on the other. Out of 44 guinea pigs subcutaneously inoculated with emulsion of street virus salivary glands, 20 developed rabies, and all the 20 showed numerous Negri bodies varying from 5μ to 20μ in diameter: a marked contrast with the guinea pigs mentioned above which had been inoculated with brain matter.

It is more difficult to secure infection in guinea pigs with salivary gland emulsion subcutaneously than with brain matter subcutaneously, a fact which other workers have noticed in dealing with dogs' salivary glands. In addition to a higher rate of infectivity brain matter has a shorter incubation period than salivary gland emulsion. The average incubation period of the 31 guinea pigs inoculated with brain matter was 16·9 days and that of the 20 inoculated with salivary gland matter was 20·7 days. We are now attempting to re-construct the Negri bodies of street virus from ordinary fixed virus by using the salivary glands of guinea pigs which have died from subcutaneous fixed virus infection. It is much more difficult, however, to obtain infection through the medium of fixed virus salivary glands than with street virus glands.

2.—On the changes which occur in the size of Negri bodies in the Hippocampus major when a strain of rabies is passed through a series of guinea pigs subdurally. (See also Bulletin 1, page 15).

The subjoined table is a continuation of Table 8, published on page 15 of Bulletin No. 1, and shows the changes in the incubation period of rabies passed through a series of guinea pigs subdurally, and the changes which occur in the size of Negri bodies to be seen in the hippocampus.

The street virus to begin with was obtained from the cortex of a naturally infected dog.

It will be observed that after the sixty-second passage Negri bodies began to disappear and could not be recognised in six instances. Mann's stain was used throughout and at least two complete sections were closely searched before "*Nil*" was recorded. It is quite possible that, had time permitted a large number of sections to be searched, tiny bodies might have been found in some or all of the negative preparations.

Number of passage.	Incubation period.	Size of Negri bodies.
41	4 days.	8μ
42	5 days.	3μ
43	5 days.	5μ
44	5 days.	3μ
45	4 days.	3μ
46	4 days.	Specimen lost.
47	4 days.	4μ
48	4 days.	8μ

Number of passage.	Incubation period.	Size of Negri bodies.
49	4 days.	3μ
50	4 days.	Specimen badly stained.
51	4 days.	2μ
52	4 days.	1μ–2μ
53	4 days.	2μ
54	5 days.	3μ
55	4 days.	3μ
56	4 days.	3μ
57	4 days.	3μ
58	4 days.	3μ
59	4 days.	3μ
60	4 days.	3μ
61	4 days.	1μ–2μ
62	5 days.	*Nil.*
63	5 days.	2μ
64	5 days.	1μ–2μ
65	4 days	2μ
66	4 days.	*Nil.*
67	4 days.	1μ
68	4 days.	*Nil.*
69	4 days.	*Nil.*
70	5 days.	2μ
71	4 days.	*Nil.*
72	5 days.	2μ
73	4 days.	2μ
74	4 days.	*Nil.*

The series was discontinued after the seventy-fourth passage as no further information could be expected from it.

3.—On Lentz's Passagewutkörper.

Lentz (Zeit. für Hygiene LXII, 1908) in 1908 described certain microscopic appearances under the name of passagewutkörper which he stated to be always present in passage rabies, *i.e.*, fixed virus, and absent in street virus and other affections.

The bodies were to be found almost exclusively in the central third of the semicircle of large ganglionic nerve cells displayed in cross sections of the hippocampus major, and were to be recognised only after the Eosin-Methylene-blue method of staining employed by Lentz, not after Mann's stain. They have no relation therefore to Negri bodies. As it is highly important to be in possession of a means of recognising sections of fixed virus hippocampus unfailingly under the microscope we at once proceeded to verify Lentz's discovery, following his method with exactitude, but we failed to find the passagewutkörper in several of the fixed virus Hippocampi examined.

In some brains the passagewutkörper were numerous and well marked, in others scanty, and in a few none at all could be demonstrated. We could not find any in street virus brains, Lentz also having observed this.

The subjoined table gives the results of the microscopic examinations.

—	Passagewutkorper.		
	Numerous.	Scanty.	Nil.
Fixed virus Rabbits	6	5	7
Street virus Guinea pigs	..	..	4
Fixed virus Guinea pigs	1	..	..

It seems evident that, when dealing with the strain of fixed virus of this Institute at any rate, one cannot depend on Lentz's method for its recognition under the microscope. The objects he describes seem to us to be degenerated nerve cells whose cytoplasm has taken on an acidophile re-action, and whose nuclear matter has broken up into basophile globules of varying number and size. Doubtless these passagewutkörper are in some way dependent on the presence of the rabic organism in the brain, but they are not constantly found, and Pinzani (Cent. für Bakt. Orig. LI, 1909) has recently shown that they are not even specific, as he has observed them in other morbid states of the brain besides fixed virus rabies. Kozewaloff (Cent. für Bakt. Orig. LII, 1909) also concludes that they are not of a parasitic nature.

SECTION II.—ON THE INFECTIVITY OF FIXED VIRUS.

1.—To ascertain in what manner the infectivity of fixed virus diminishes when preserved in glycerine.

The spinal cords of fixed virus rabbits were divided into numerous pieces, each of which was carefully weighed. The alternate pieces were preserved in the ice box immersed in the three following fluids:—

(1) Pure glycerine.
(2) 50 per cent. glycerine in distilled water.
(3) 25 " " " "

The comparative inoculations were made with adjacent pieces of cord from the same region, so as to eliminate the error that might be introduced if lumbar cord in pure glycerine were to be compared with cervical cord in 50 per cent. glycerine.

Table 1 *shows the results of the total number of inoculations performed.*

Cord preserved in	Number of rabbits inoculated subdurally.	Rabies.	Survived.	Died otherwise.	Average incubation period.
Pure glycerine	54	27	23	4	9·3 days.
50 per cent. glycerine ..	46	37	8	1	7·7 ,,
25 per cent. glycerine ..	16	9	7	..	12·0 ,,

Table 2 *shows the results obtained with the different dilutions of the cord substance employed.*

Dilutions of emulsions of fixed virus cord.	Pure Glycerine.			50 per cent. Glycerine.			25 per cent. Glycerine.		
	Rabies.	Survived.	Died otherwise.	Rabies.	Survived.	Died otherwise.	Rabies.	Survived.	Died otherwise.
1 in 50 ..	1	..	..	1	..	..	..	..	..
,, 100 ..	4	1	..	4	..	..	1	..	..
,, 250 ..	5	4	1	5	..	1	3	2	..
,, 500 ..	9	5	1	12	..	..	2	3	..
,, 1,000 ..	6	7	..	9	2	..	3	1	..
,, 2,000 ..	2	4	2	5	4	..	..	1	..
,, 4,000 ..	..	1	..	..	1	..	..	..	..
,, 5,000 ..	..	1	..	1	1	..	..	..	..
Total ..	27	23	4	37	8	1	9	7	..

Table 3 *shows the effect on the incidence of rabies of the time during which the fluids were in contact with the virus.*

Number of days the virus was preserved.	Pure Glycerine.			50 per cent. Glycerine.			25 per cent. Glycerine.		
	Rabies.	Survived.	Died otherwise.	Rabies.	Survived.	Died otherwise.	Rabies.	Survived.	Died otherwise.
2 days ..	2	6	3	7	3	1	2	2	..
4 ,, ..	7	2	1	6	1	..	3	..	..
7 ,, ..	..	4	..	6	2	..	..	..	..
10 ,, ..	4	3	..	4	..	..	2	1	..
14 ,, ..	5	6	..	7	1	..	1	2	..
20 ,, ..	6	1	..	4	..	..	1	2	..
32 ,, ..	3	1	..	3	1	..	..	..	..
Total ..	27	23	4	37	8	1	9	7	..

The figures show that there is undoubted diminution of virulence due to the action of glycerine, whether pure or diluted with salt solution: but no relation between the length of time the material was kept and its virulence is apparent.

The chief point brought out is the much greater proportion of infections obtained with the material preserved in 50 per cent. glycerine than with that preserved in pure glycerine (80 per cent. against 46 per cent.), 25 per cent. glycerine, however, seems to be a worse medium than 50 per cent. glycerine, giving an infection rate of 56 per cent. though this number is based on fewer figures. The average incubation period is in conformity, being 7·7 days for the material prescribed in 50 per cent. glycerine, 9·3 days for pure glycerine and 12 days for 25 per cent. glycerine.

The conclusions to be drawn are—

(1) that fixed virus deteriorates, though to an unknown degree, when kept even for as short a time as 48 hours in contact with pure glycerine at the temperature of the ice box, 5° C to 10° C;

(2) that 50 per cent. glycerine is a better preservative than either pure or 25 per cent. glycerine and that this medium should therefore be selected for the preservation of brains sent from a distance to an antirabic institute for the biological test.

2.—On the susceptibility of Monkeys to Rabies.

(1) Of 10 monkeys, inoculated once subcutaneously in the abdomen with street virus brain matter from dogs and rabbits in varying quantities—

4 developed rabies within 12 weeks. Subdural sub-inoculations were made in guinea pigs from the brains of two of these monkeys, one was positive with an incubation of 9 weeks and the other negative.

2 died otherwise after 12 weeks.

4 survived more than 12 weeks, but succumbed to street virus injected intraocularly, showing that they had not been immunised.

(2) Of 7 monkeys, inoculated once subcutaneously in the abdomen with fresh fixed virus brain matter from rabbits, 1/10,000 of the body weight being injected in each case, all survived up to 10 weeks—

4 died otherwise between 10 and 13 weeks.

(No Negri bodies, sub-inoculations in guinea pigs negative.)

3 succumbed afterwards to inoculations of street virus, two intraocular and one subcutaneous, showing that no immunity had been established.

(3) Of 5 monkeys, inoculated once subcutaneously in the abdomen with fresh fixed virus brain substance from rabbits, 1/1,000 of the body weight being injected in each case—

all survived up to 10 weeks.

2 died otherwise after 10 weeks.

3 succumbed afterwards to street virus inoculations, two subcutaneous and one intraocular, showing that no immunity had been established.

(4) Of 10 monkeys, inoculated daily for 14 days subcutaneously in abdomen with fresh fixed virus brain substance from rabbits, 1/10,000 of the body weight being injected altogether—

4 survived from 10 to 19 weeks.

(When dead there was no microscopic evidence of rabies and all sub-inoculations in guinea pigs were negative.)

2 died before 10 weeks.

(No evidence of rabies, both sub-inoculations in guinea pigs were negative.)

1 survived 12 weeks and then succumbed 11 days after intraocular injection of street virus (Negri bodies plentiful).

1 survived 12 weeks and succumbed 40 days after intraocular injection with street virus.

(No Negri bodies, sub-inoculation in guinea pig negative.)

2 survived 12 weeks and succumbed two months after subcutaneous inoculation with street virus.

(No Negri bodies, sub-inoculation in guinea pigs negative.)

(5) Of 10 monkeys, inoculated daily for 14 days subcutaneously in abdomen with fresh fixed virus brain substance from rabbits, 1/1,000 of the body weight being injected altogether—

all survived 10 to 18 weeks.

6 died later. (No Negri bodies, sub-inoculations in guinea pigs negative.)

2 were afterwards inoculated intraocularly with street virus and succumbed in 15 and 33 days.

(Negri bodies present. No sub-inoculations made.)

2 were afterwards inoculated subcutaneously with street virus and succumbed in 29 and 46 days.

(Negri bodies present, sub-inoculations negative.)

Conclusions.

(1) That monkeys survive subcutaneous injection of fresh fixed virus rabbit brain in abdomen—

(*a*) in single doses up to 1/1,000 of body weight,

(*b*) in daily doses for 14 days, up to 1/1,000 of body weight being injected in all.

(2) That little or no immunity is established against subsequent subcutaneous or intraocular inoculation of street virus, either by single injections of fresh fixed virus or by daily injections for 14 days.

(3) That supposing that monkeys which die after fixed virus inoculation actually die from rabies, a supposition which is not supported by the symptoms observed, it is exceedingly difficult to infect guinea pigs by subdural inoculation from their brains: in fact in no instance were sub-inoculations successful in this respect.

(4) That about 40 per cent. of monkeys succumb to subcutaneous inoculation of street virus brain matter.

(5) That Negri bodies cannot always be demonstrated in sections of the brain of monkeys after either subcutaneous or intraocular inoculation of street virus.

(6) That it is extremely difficult to infect guinea pigs even by subdural inoculation from the brains of monkeys which have succumbed from street virus: in fact we only succeeded once with an incubation period of 62 days.

(7) That monkeys are moderately susceptible to street virus inoculated subcutaneously, but resistant to fixed virus, and that, even when they do succumb to street virus and Negri bodies are found in their brains, the virus is so greatly attenuated that it does not readily infect guinea pigs subdurally. (*Vide* attenuation by passage through birds, Bulletin 1, page 21.)

3.—To determine whether any difference existed in the infectivity of three strains of rabies virus in three different species.

The strains used were—

(1) Laboratory rabbit virus, originally derived from a dog, 248th subdural passage in rabbits.

(2) Guinea pig virus, originally derived from a dog, 29th subdural passage in guinea pigs.

(3) Dog virus, originally derived from a dog, 19th subdural passage in dogs.

The following table shows the effect of each of these three strains as regards the length of the incubation period and the duration of the disease when inoculated subdurally :—

Animal inoculated subdurally.	Incubation period in days.	Death Days.	Virus used.
Guinea pig	5 to 6	7 to 8	Laboratory rabbit virus.
Rabbit	6	8 to 9	,, ,,
Monkey	9	11	,, ,,
Guinea pig	5	6 to 7	Guinea pig virus.
Rabbit	5	6	,, ,,
Monkey	5 to 7	16	,, ,,
Guinea pig	10	10½	Dog virus
Rabbit	8	13½	,, ,,
Monkey	11	11½	,, ,,

It appears from the table that dog street virus passed through 29 guinea pigs is more rapid in its action than dog street virus passed through 248 rabbits or than dog street virus passed through 19 dogs. It must, however, be remembered that a different strain was used for each series and they might have differed in activity in the first place. In each case the virus used was fixed, *i.e.*, the rabbit strain had an incubation of 6 days in rabbits, the guinea pig virus, an incubation of 5 days in guinea pigs and the dog virus, an incubation of 9 days in dogs.

There was no difference in virulence in the three strains; they all killed surely, but they differed in activity, the dog strain being the least active. As we have shown before (Bulletin 1, page 16), rabies does not die out when inoculated subdurally into a succession of dogs but becomes fixed. If ordinary street dogs be used for the series, a certain number of passages will fail owing to the insusceptibility of the subjects. One concludes therefore that dogs are less susceptible to rabies than either rabbits or guinea pigs, and probably the virus they provide is somewhat less active than that obtained from rabbits and guinea pigs, and, probably, also rats. Apparently the only reason why rabies does not spread in nature amongst such animals as rabbits or guinea pigs is because it is not their habit to bite, as it is the habit of dogs, and rabies, if introduced, would speedily die out in consequence. The virus finds its way to the salivary glands of guinea pigs just as it does in dogs.

4.—To ascertain if protective inoculations of fixed virus are of more value round the site of infection with street virus than at a distance.

(1) Six guinea pigs were inoculated subcutaneously in the abdomen with first passage street virus brain. On the next day protective subcutaneous injections with fixed virus were begun. In all, fourteen injections, amounting to 1/10,000 of the animal's weight of fresh fixed virus, were given in a circle round the point infected with street virus.

Three of the six survived. Of the remaining three—

No. 84 died on 14th day.
,, 105 ,, 15th ,,
,, 181 ,, 17th ,,

No. 84 showed no Negri bodies: no sub-inoculation made.

No. 115 ,, ,, a guinea pig inoculated subdurally with its brain showed Negri bodies to 3μ in hippocampus and to 6μ in cerebellum.

No. 181 showed tiny Negri bodies: no sub-inoculation made.

As we have shown elsewhere (page 1), subcutaneous inoculation of street virus brain substance in guinea pigs in a large proportion of cases does not produce Negri bodies in the brain, yet these bodies may reappear if subdural passages be made from the brain of the animals which have succumbed to subcutaneous inoculation. The fact that large and typical Negri bodies were absent from the brains of guinea pigs 84, 115

and 181 is therefore no proof that they did not die from the street virus infection and the fact that Negri bodies were recovered in a sub-passage from No. 115 makes it clear that that animal at least died from street virus infection. Sub-passages were not made from No. 84 and No. 181, so, it remains uncertain whether they succumbed to street virus or fixed virus infection.

(2) Six other guinea pigs were inoculated subcutaneously in the back with first passage street virus brain.

On the next day protective subcutaneous injections with fixed virus were begun. In all fourteen injections, amounting to 1/10,000 of the animal's weight of fresh fixed virus were given in the abdomen at a distance from the street virus infection unless death occurred before the end.

One of the six survived. Of the remaining five—

No. 80	died on	14th	day.
" 5A	"	13th	"
" 91	"	13th	"
" 65	"	14th	"
" 50	"	12th	"

No. 80 showed no Negri bodies, no sub-inoculation made.
No. 5A showed Negri bodies to 1·5μ "
No. 91 showed Negri bodies to 5·0μ, in sub-passage Negri bodies to 4μ.
No. 65 showed Negri bodies to 3·0μ, no sub-inoculation made.
No. 50 showed no Negri bodies, no sub-inoculation made.

Whether Negri bodies were found or not it is probable that all these five animals succumbed to the street virus infection.

It follows either that street virus infects guinea pigs with greater certainty and more rapidly when inoculated beneath the skin of the back than it does when inoculated beneath the skin of the abdomen, or else that fixed virus injected round the site of the street virus infection has greater protective power than when injected at a distance from the infected part.

The experiments merely indicate the possibility that protective injections might have more effect if a local immunity in the neighbourhood of the point infected were to be aimed at instead of a general immunity. They prove nothing except that guinea pigs are far too susceptible to both street virus and fixed virus subcutaneously to be suitable animals for this class of experiment.

The idea seems to be gaining ground that, after a natural infection with street virus through the bite of a rabid animal, the virus frequently becomes rapidly disseminated by the blood and lymph streams, and does not merely lie in the wound and travel slowly to the central nervous system along the nerve trunks. If this be so, there would be little gained by endeavouring to excite a more intense immunity of the tissues in the neighbourhood of the point of infection.

5.—Is the virus of rabies destroyed at the point of introduction or does it lie latent in the subcutaneous tissue?

(1) Each of two guinea pigs was inoculated subcutaneously in the midline of the abdomen with 0·3 gramme fixed virus rabbit brain suspended in 1 cc. salt solution.

Six days later they were killed and the subcutaneous tissue at the site of the inoculation was dissected out. In one animal there was no trace of the inoculum; in the other there was a nodule of the size of a pea. The tissues removed by dissection were pounded up and inoculated subdurally into another guinea pig, which survived.

(2) A tiny nodule was removed from the subcutaneous tissue at the site of inoculation of a rabbit which had succumbed to fixed virus injected subcutaneously. It was emulsified and inoculated subdurally into a guinea pig, which survived.

Microscopic sections of these subcutaneous nodules show them to consist mainly of a collection of eosinophile leucocytes.

It must be concluded that fixed virus, at any rate, if introduced into the subcutaneous tissues, is rapidly destroyed and that after six days a sufficient quantity cannot be collected at the site of inoculation to infect a guinea pig subdurally. In spite of this the subcutaneous inoculation may cause rabies.

The two guinea pigs having been killed on the sixth day after the inoculation had shown, as is only natural, no symptoms of rabies. It is, therefore, possible that they would not have succumbed to rabies if they had been allowed to live longer and that the absence of infective matter in the nodules was due to the inherent protective power of their tissues which had destroyed the virus.

This possibility is excluded in the case of the rabbit which actually died of rabies, and the fact lends support to the view that it is the virus which is rapidly disseminated throughout the body, until some of it finds a suitable location in the central nervous system, which is the real starting point of the disease.

The peculiar assemblage of eosinophile cells at the point of inoculation is noteworthy.

6.—To ascertain the difference in infectivity of the brain and lumbar cord of subdurally inoculated rabbits (Laboratory fixed virus), *(a)* which have presented the normal incubation period of 6 to 7 days and *(b)* which have had a prolonged incubation period.

	Rabbit.	Date of inoculation.	Material used.	Dilution used.	Position of inoculation.	Incubation period.
Series 1.	107 ..	1st June 1908.	Whole brain of fixed virus rabbit 134. Passage 236. Incubation 6 days. Died 10th day.	1 in 5	Subdural.	5 days.
	183 ..	,,		1 in 500	,,	6 ,,
	104 ..	,,		1 in 5,000	,,	7 ,,
	6 ..	,,		1 in 50,000	,,	Survived.
	184 ..	1st June 1908.	Whole lumbar cord of same rabbit.	1 in 200	Subdural.	6 days.
	30 ..	,,		1 in 1,000	,,	Survived.
	69 ..	,,		1 in 5,000	,,	9 days.
Series 2.	38 ..	1st June 1908.	Whole brain of fixed virus rabbit 100. Passage 236. Incubation 6 days. Died 10th day.	1 in 5	Subdural.	7 days.
	35 ..	,,		1 in 500	,,	7 ,,
	74 ..	,,		1 in 5,000	,,	8 ,,
	91 ..	,,		1 in 50,000	,,	Survived.
	47 ..	1st June 1908.	Whole lumbar cord of same rabbit.	1 in 200	Subdural.	7 days.
	128 ..	,,		1 in 1,000	,,	7 ,,
	125 ..	,,		1 in 5,000	,,	Survived.
Series 3.	182 ..	1st June 1908.	Whole brain of fixed virus rabbit 81 which had been subdurally inoculated with 1 in 5,000 fixed virus lumbar cord. Incubation 21 days. Died 24th day.	1 in 5	Subdural.	7 days.
	85 ..	,,		1 in 500	,,	7 ,,
	92 ..	,,		1 in 5,000	,,	7 ,,
	98 ..	,,		1 in 50,000	,,	Survived.
	110 ..	1st June 1908.	Whole lumbar cord of same rabbit.	1 in 200	Subdural.	6 days.
	108 ..	,,		1 in 1,000	,,	7 ,,
	172 ..	,,		1 in 5,000	,,	Survived.

The quantity of the emulsion inoculated was in each case 0·2 cc. as nearly as possible.

It appears from these three series that there is no difference in the relative infectivity of the lumbar cord and the brain whether the incubation period of the rabbit has been 6 or 21 days, that an emulsion of the whole brain has a higher degree of infectivity than an emulsion of the whole lumbar cord (*vide* pages 10 to 12, Bulletin 1, 1908), and finally that there need be no hesitation in using material for patients from a rabbit which has developed rabies only after a prolonged incubation period, since the brain and lumbar cord of such an animal show precisely the same degree of infectivity as the brain and lumbar cord derived from a rabbit which has developed rabies after the usual incubation period of 6 to 7 days.

SECTION III.—HAEMOLYTIC DATA.

1.—To ascertain whether lysins and agglutinins are formed as a result of subcutaneous injections of serum free from erythrocytes.

Two goats were selected and were injected at intervals, one H with fresh sheep serum, the other G with sheep serum heated to 55° C for 30 minutes. The serum of neither goat either dissolved or agglutinated sheep erythrocytes before treatment.

GOAT G.

Heated sheep serum	... ...	15 cc. on 9th April 1909.
" "	... ...	18 cc. " 14th " "
" "	... ...	25 cc. " 23rd " "
" "	... ...	30 cc. " 4th May "

GOAT H.

Fresh sheep serum	... ...	15 cc. on 9th April 1909.
" "	... ...	18 cc. " 14th " "
" "	... ...	25 cc. " 23rd " "
" "	... ...	30 cc. " 4th May "

On 14th May 1909 the goats were bled and their serums tested with the following results :—

GOAT G SERUM.

100 % concentration, or 39 parts to 1 part of sheep erythrocytes, gave "nearly complete" lysis. Slight agglutination.

GOAT H SERUM.

12 % concentration or 5 parts of serum to one part sheep erythrocytes gave "complete" lysis. Slight agglutination.

Assuming that the two goats re-acted in an equal degree to the injections, which is impossible of proof, it is evident that the injection of fresh serum procures the formation of a specific lysin over 7 times more powerful than the lysin obtained by the injection of heated serum.

Both heated and unheated serums cause the production of small quantities of erythrocyte agglutinin.

N.B.—The serum injected was not centrifugated before use and so a few erythrocytes probably remained in suspension though the liquid was clear to the eye. In any case, if the formation of the anti-sheep lysin was due to these cells and not entirely to the serum it was obtained by the injection of a remarkably small number of them.

2.—Some points on the relation of quinine salts and arsenic to hæmolysis.

(1) Quinine sulphate 0·1 per cent. dissolved in NaCl 0·85 per cent. does not dissolve sheep erythrocytes.

The specific serum of goat B, 0·05 cc. of which dissolved 0·05 cc. sheep erythrocytes completely and 0·01 cc. of which gave no lysis with the same quantity of erythrocytes, was neither assisted nor prevented in its action by the presence of 0·1 per cent. quinine sulphate or quinine hydrochloride in the mixture.

The following results were obtained :—

Quinine hydrochloride in 0·85 °/₀ NaCl.	*Sheep erythrocytes.*	*Degree of lysis.*
1·0 °/₀	0·05 cc.	Complete.
0·2 °/₀	,,	Trace.
0·02 °/₀	,,	Nil.
	Man erythrocytes.	
1·0 °/₀	0·05 cc.	Complete.
0·2 °/₀	,,	Trace.
0·02 °/₀	,,	Nil.
Quinine sulphate in 0·85 °/₀ NaCl.		
0·1 °/₀	,,	Trace.
0·005 °/₀	,,	Nil.

It is evident that the erythrocytes of both man and sheep are permeable to quinine salts and that these in certain concentrations will cause the hæmoglobin to diffuse out of the cells. Quinine sulphate is not sufficiently soluble in neutral saline solutions to enable a solution of much higher concentration than 0·1 per cent. to be prepared, and this is not sufficient to cause more than a trace of lysis. It is practically certain therefore that salts of quinine can never be present in the blood in sufficient concentration to cause any lysis of themselves and that the hæmoglobinuria of blackwater fever witnessed after a dose of quinine, whether sulphate or hydrochloride, cannot be due to the action of the quinine alone; some other factor must be superadded. It is possible that even a trace of quinine in the blood, since it penetrates the erythrocytes, may render them less resistant to the peculiar lysin which gives rise to hæmoglobinuria. Erythrocytes are impermeable to the sulphates of sodium and potassium and if they are suspended for some time in solutions of Na_2SO_4 they are not altered in their subsequent behaviour either to a specific lytic serum or to hypotonic solutions.

It is not likely therefore that the presence of quinine sulphate in the blood, as sulphate, makes the slightest difference in the resistance of the erythrocytes.

(2) Arsenious oxide is very sparingly soluble in water. In concentrations of 0·1 per cent. to 0·001 per cent. it neither increases nor diminishes the resistance of either goat, sheep or man erythrocytes to lysis by a specific serum or by hypotonic solutions. It is probable therefore that the presence of minute quantities of arsenic in the blood would have no influence on the appearance of hæmoglobinuria. Dr. Gunn (Brit. Med. Journ., Volume II, 1908, page 145) states that he has found that arsenic increases the resistance of erythrocytes to lysis by water. His findings being at variance with ours we carefully repeated his experiments and neither by his method nor by ours could we find any increase of resistance indicated.

3.—On the transference of hæmolytic power from parent to offspring.

Goat B serum had been powerfully hæmolytic to sheep erythrocytes for several months as a result of periodical subcutaneous injections of sheep erythrocytes.

On 10th July 1908 goat B had a kid. When this kid was 7 days old its opalescent serum in a concentration of 8 per cent. in 2 cc. of 0·85 per cent. NaCl completely dissolved 0·05 cc. washed sheep erythrocytes. On 13th August 1908, when the kid was 41 days old, it required 33 per cent. of its serum to give complete lysis.

When 72 days old	33 per cent. serum gave ...	...	"not complete" lysis
When 100 ,,	66 ,, ,, ...	...	"slight" ,,
When 154 ,,	100 ,, ,, ...	...	"no" ,,

So the lytic power of the serum of this kid passed away entirely within 100 to 154 days of its birth.

During this period goat B was suckling the kid. Her fresh milk dissolved sheep erythrocytes only slightly, and after heating to 55° C for 30 minutes not at all. When the fresh milk was complemented by the addition of the serum of a normal sheep complete lysis was obtained. The milk of goat B therefore contained plenty of amboceptor for sheep erythrocytes but very little complement.

Goat B had two more kids on 5th January 1909. The serum of one of these kids was tested against 0·05 cc. sheep erythrocytes at intervals with the following results :—

Kid	7 days old	7·5 per cent. serum	... ...	complete lysis.
„	35 „	7·5 „ „	... ...	„ „
„	63 „	12·0 „ „	... ...	„ „
„	91 „	32·0 „ „	... ...	„ „
„	126 „	82·0 „ „	... ...	„ „
„	147 „	100·0 „ „	... ...	slight „
„	182 „	100·0 „ „	... ...	no „

The serum of this kid, born on 5th January 1909, contained more lysin than that of the first, born on 10th July 1908, probably because goat B had received several more injections of sheep erythrocytes in the interval, but it all disappeared within approximately the same period.

To ascertain whether the lysin is conveyed by the mother's milk or by the placental blood experiments were made with another goat, goat A.

Goat A had two kids on 10th July 1908. The serum neither of goat A nor of her two kids lysed sheep erythrocytes.

Goat A then received injections of sheep erythrocytes.

On 26th July 1908, 8 per cent. of goat A's serum lysed 0·05 cc. sheep erythrocytes completely. While her serum contained lysin for sheep erythrocytes she was suckling her kids, but no lysis could be obtained with the serum of either kid ; so it appears that a specific lysin is conveyed to offspring by placental blood and not by milk.

4.—To find if there is much variation from day to day in the resistance of the erythrocytes of a healthy animal to lysis by hypotonic solutions of sodium chloride.

The erythrocytes of a normal goat were tested daily for 14 days.

Method.—The blood was received into a solution containing 1 per cent. sodium citrate and ·85 per cent. sodium chloride, centrifugated and washed twice in ·85 per cent. salt.

A series of test tubes was prepared containing gradated percentages of salt, 2·5 cc. in each, the difference between any two adjacent tubes being equivalent to ·017 per cent. NaCl. Into each tube was inserted by a calibrated pipette ·05 cc. of the washed erythrocytes which were well distributed by immediate shaking. The tubes were afterwards shaken up at intervals, left at room temperature for an hour and then kept in the ice chest till next morning, when the degree of the lysis was judged.

Percentage of NaCl showing "trace" lysis—	
Mean of the 14 days	·753 per cent.
Highest point above mean	·029
Lowest point below mean	·039
Variation in "trace" lysis	·068 per cent. NaCl.
Percentage of NaCl showing "complete" lysis—	
Mean of the 14 days	·598 per cent.
Highest point above mean	·031
Lowest point below mean	·059
Variation in "complete" lysis	·085 per cent. NaCl.
Range between means of trace and complete lysis	·155 per cent. NaCl.

It appears therefore that the daily variations of the resistance of the erythrocytes of a healthy animal to lysis by hypotonic salt solution are very small.

The daily variations in the percentage of chlorides in the serum of this goat were also estimated (for method *vide* Bulletin 1, page 30).

Mean of 14 days	·64 per cent. NaCl.
Highest observation	·67 per cent. ,,
Lowest ,, ...	·61 per cent. ,,

Chart 1 shows the daily variations in the resistance of the erythrocytes of a healthy goat to lysis by hypotonic salt solution for a period of 14 days together with the variations in the percentage of chlorides contained in the serum of the same animal.

The range between the first noticeable "trace" of lysis and "complete" lysis seems to remain about the same; that is to say, as far as can be judged by this somewhat rough visual method, if on any particular day there are more erythrocytes in the circulation which are less resistant to the highest percentages of salt employed there are also fewer which are resistant to the lowest percentages of salt employed.

The range between the "trace" and "complete" points appears to be much the same in all the species of animals so far tested, though each species occupies a definite position on the percentage scale, but into this matter we hope to enter more fully in a subsequent issue.

Suffice it to say at present that the vast majority of the erythrocytes of any healthy animal are lysed completely by lowering the tonicity of the suspending fluid to a certain point; that comparatively few are more resistant or less resistant than the majority; that variations from the mean are measured by actually small differences in the tonicity of the suspending fluid and that the fluctuations in resistance from day to day seem to effect the whole number of the erythrocytes.

There appears to be no obvious relation between the fluctuations in resistance of the erythrocytes and the percentage of chlorides present in the serum. (*Vide* chart 1.)

We have not touched upon variations in resistance resulting from disease but a careful experiment made by dieting two puppies seems to show that, as long as no actual organic disease is engendered, unsuitable diet carried nearly to the extent of arrest of growth has no influence on the resistance of the erythrocytes.

Two healthy puppies, 3 months old, from the same litter were selected and the lytic points of their erythrocytes determined. One was then fed on an exclusively meat diet, the other on an exclusively vegetable diet. During the period of the experiment "Meat" increased in weight by 66 per cent. while "Vegetable" only increased by 5 per cent.

After 21 days dieting the lytic points of the erythrocytes of both animals were precisely the same as they were before the dieting began. After 32 days there was a slight fall in resistance in both animals but it was the same for each.

	Before dieting.	After 21 days.	After 32 days.
"*Meat*"—			
Weight in grammes ...	2,300	2,970	3,830
"Trace" lysis ...	·510 per cent.	·510 per cent.	·527 per cent. NaCl.
"Complete" lysis ...	·374 ,,	·374 ,,	·391 ,, ,,
"*Vegetable*"—			
Weight in grammes ...	2,600	2,720	2,730
"Trace" lysis ...	·510 ,,	·510 ,,	·527 ,, ,,
"Complete" lysis ...	·374 ,,	·374 ,,	·391 ,, ,,

At the end of the experiment it was found that 62 per cent. of "Meat's" serum in a total volume of 2·0 cc. was required to completely dissolve 0·05 cc. washed human erythrocytes and 52 per cent. of "Vegetable's" serum.

It may be concluded, therefore, that the serum of an ill-nourished but otherwise healthy animal may be expected to contain as much lysin as that of a well nourished animal and that the erythrocytes of healthy animals of the same species will not be found to vary as regards their lytic points beyond the normal range as a consqeuence of poor feeding.

CHART. 1.

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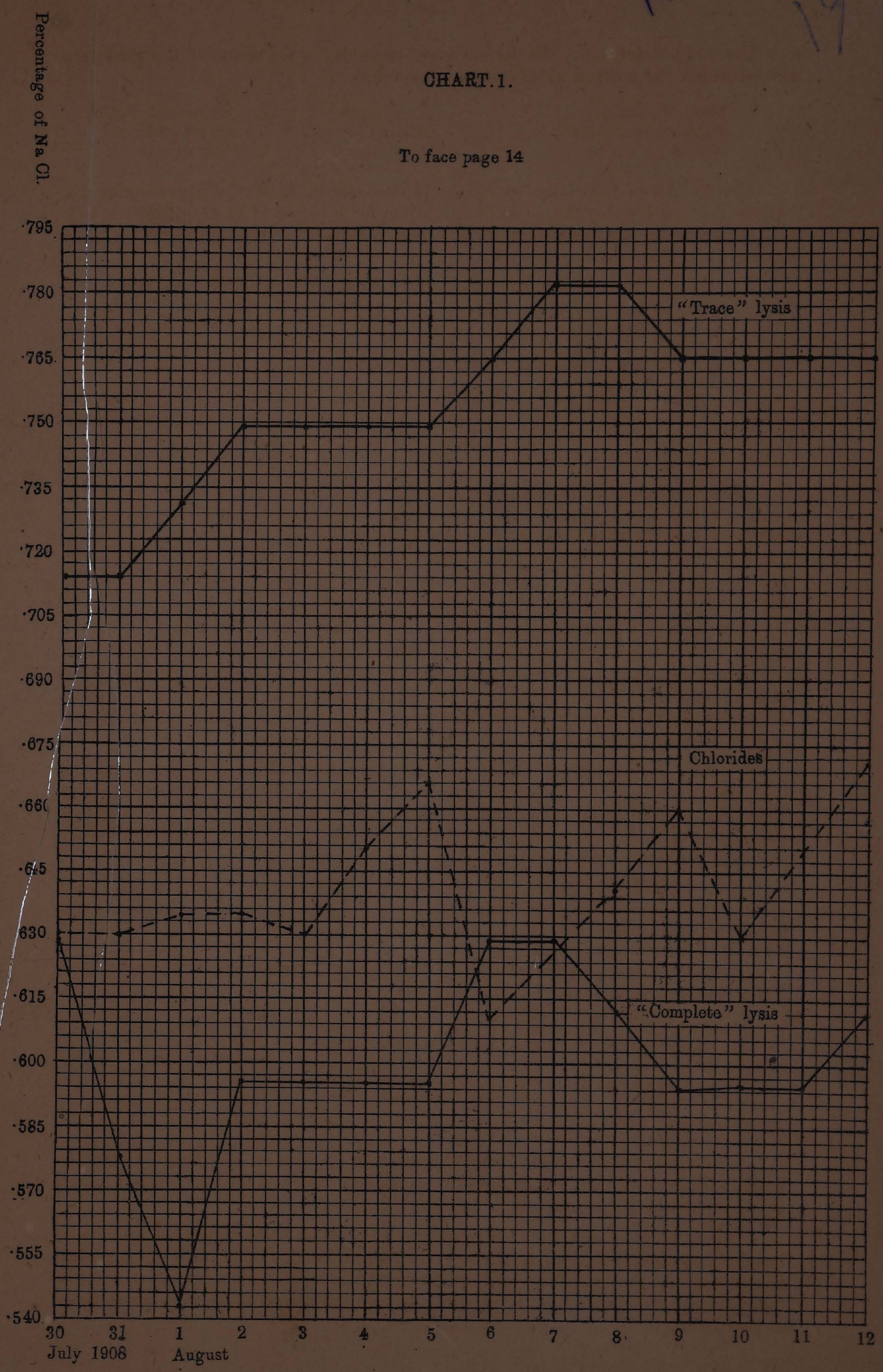

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CHART 2

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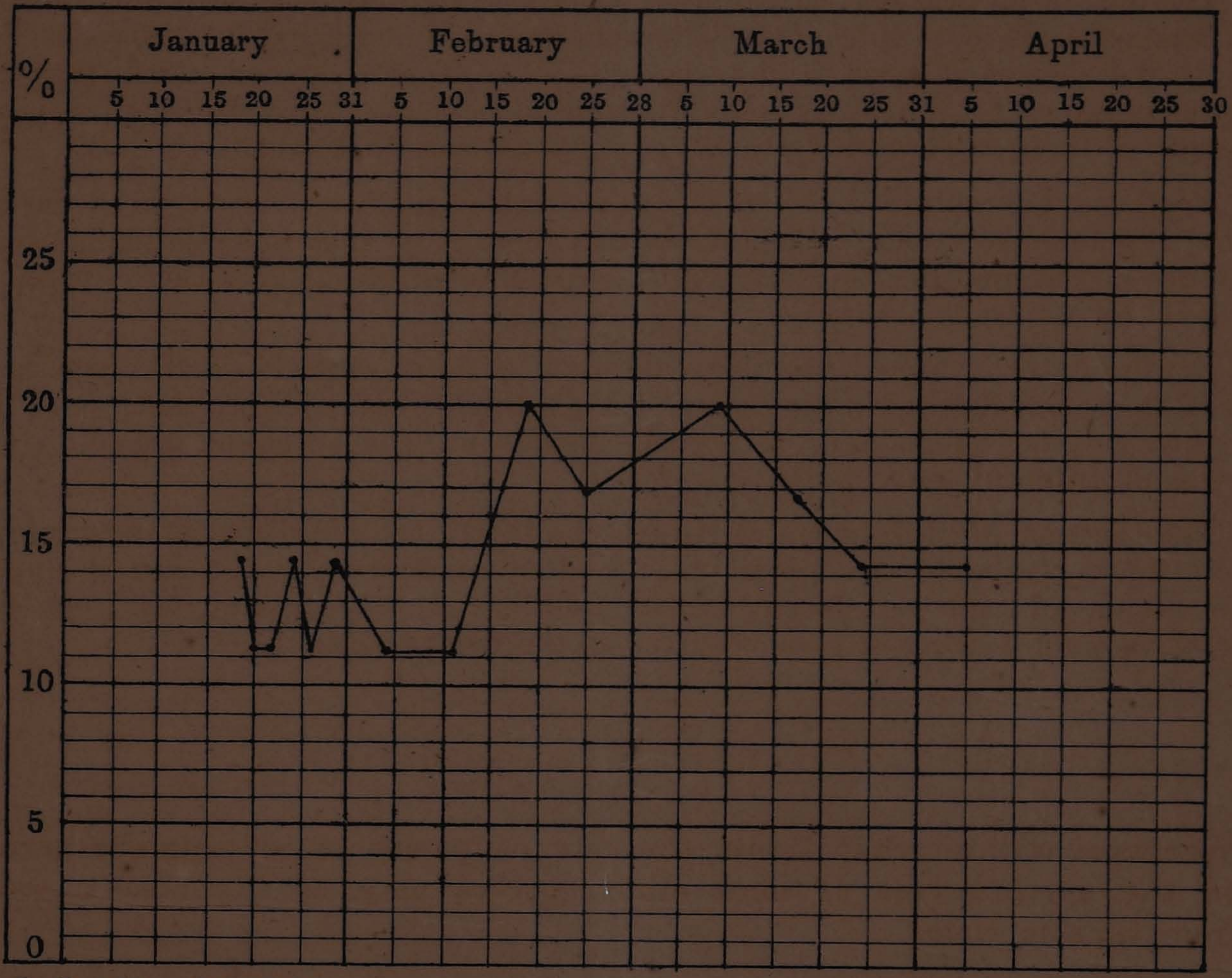

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5.—Some points on the variations of the erythrocytes of individuals of the same species to lysis by hypotonic solutions and by specific serum.

(1) Having first ascertained the tonicity of the blood of a rabbit, expressed in terms of NaCl, a comparison was made between erythrocytes suspended in their plasma and washed erythrocytes suspended in 0·85 per cent. NaCl to determine whether the lysis by hypotonic salt solution began and ended at the same points in each case. No difference in the points could be observed, so it may be concluded that erythrocytes are lysed by hypotonic solutions of exactly equivalent strengths whether the dilution be made of the whole blood, due allowance being made for the normal tonicity of the plasma, or whether washed erythrocytes freed from plasma be used.

(2) The serum of goat B, which lysed sheep erythrocytes specifically, was tested with the washed erythrocytes from six different sheep. Practically no difference was observed in the degree of lysis in the six series, so it may be concluded that the erythrocytes obtained from healthy individuals of any species do not differ appreciably in the degree of their susceptibility to lysis by a specific lytic serum.

(3) Erythrocytes obtained from the same six sheep were lysed in different degrees by hypotonic solutions, which shows that there is no apparent relation between the resistance of the erythrocytes to lysis by hypotonic solutions and their susceptibility to lysis by a specific serum.

(4) Erythrocytes obtained from six different rabbits were lysed in each case to precisely the same degree by the serum of one normal goat showing that, as in the case of the specific hæmolysin, a normal hæmolysin has an equal action on the erythrocytes of different individuals.

(5) The serums of different individuals, however, contain different amounts of hæmolysin for erythrocytes from one individual of another species. For example, using the serums of twelve different normal rabbits and the erythrocytes from one normal goat varying degrees of lysis occurred in the twelve series.

6.—To ascertain within what limits the solvent power of an animal's serum on the erythrocytes of some other species may be expected to vary from time to time.

Having already found that erythrocytes taken from different healthy animals are lysed in the same degree by a serum from another species we have only one composite variable to measure in determining the relative quantity of lysin present from day to day in a serum.

The serum of a normal goat was tested every second day from January 18th to 28th and then weekly from February 4th, April 5th, 1909, against washed rabbit erythrocytes.

Between January 18th to 28th, a concentration of 17 per cent. to 22 per cent. of serum in a total volume of 2 cc. fluid was required to completely dissolve 0·05 cc. of rabbit erythrocytes, *i.e.*, from 7 to 9 parts of serum to 1 part of erythrocytes.

Between February 4th and April 5th from 12 per cent. to 22 per cent. of serum was required, *i.e.*, from 5 to 9 parts of serum to 1 part of erythrocytes.

During the observation period of $2\frac{1}{2}$ months, the serum of this goat varied so much in its contained lysin that, on some occasions, only 5 parts of serum were required to dissolve 1 part of erythrocytes and on other occasions 9 parts of serum, or nearly double the quantity.

Chart 2 shows the rabbit-erythrocyte-lysin content of the serum of a goat between January 18th and April 5th, 1909.

7.—On the influence of sodium citrate on hæmolysis.

To ascertain whether plasma contains a greater amount of normal hæmolysin than serum allowed to separate from blood drawn at the same time, the blood of a new goat was tested against rabbit erythrocytes. Four series of tubes were put up with a result such as shown in table 1 :—

TABLE 1.

Contents of each tube.	Defibrinated goat blood.	Citrated goat blood (citrate 1 per cent.)	Goat serum 24 hours old.	Goat serum 48 hours old.
* Serum .. .. 1·95 cc. ·85 per cent. salt ·00 cc. Rabbit R.B.Cs. .. ·05 cc.	Complete (1 per cent. NaCl).	Nearly complete (1·6 per cent. NaCl).	Complete.	Complete.
Serum .. .. ·65 cc. Salt .. .. 1·30 cc. R.B.Cs. .. .. ·05 cc.	Complete.	Nearly complete (1·5 per cent. NaCl).	Complete.	Complete.
Serum .. .. ·45 cc. Salt .. .. 1·50 cc. R.B.Cs. .. .. ·05 cc.	Complete.	Partial (1·0 per cent. NaCl).	Complete.	Nearly complete.
Serum .. .. ·35 cc. Salt .. .. 1·60 cc. R.B.Cs. .. .. ·05 cc.	Complete (0·85 per cent. NaCl).	Slight (0·97 per cent. NaCl).	Nearly complete.	Partial.
Serum .. .. ·25 cc. Salt .. .. 1·70 cc. R.B.Cs. .. .. ·05 cc.	Nearly complete.	Trace.	Partial.	Partial.
Serum .. .. ·2 cc. Salt .. .. 1·75 cc. R.B.Cs. .. .. ·05 cc.	Partial.	Trace.	Partial.	Slight.
Serum .. .. ·15 cc. Salt .. .. 1·80 cc. R.B.Cs. .. .. ·05 cc.	Slight.	Trace.	Slight.	Slight.
Serum .. .. ·1 cc. Salt .. .. 1·85 cc. R.B.Cs. .. .. ·05 cc.	Trace.	*Nil.*	Trace.	Trace.
Serum .. .. ·05 cc. Salt .. .. 1·9 cc. R.B.Cs. .. .. ·05 cc.	*Nil.*	*Nil.*	*Nil.*	*Nil.*
Salt .. .. 1·95 cc. R.B.Cs. .. .. ·05 cc.	*Nil.*	..	..	..

* NOTE.—"Serum" stands for centrifugated defibrinated blood, centrifugated citrated blood or serum as the case may be. The figures in brackets indicate the tonicity of the contents of the tubes in terms of NaCl.

This experiment showed that the following quantities were equivalent in their power of completely dissolving 0·05 cc. washed rabbit R.B.Cs. :—

Defibrinated goat blood (fresh) ... ... 0·35 cc.
Goat serum, 24 hours old ... ... 0·45 cc.
Do. 48 do. ... ... 0·65 cc.

But even 1·95 cc. goat blood to which 1 per cent. sodium citrate had been added failed to dissolve completely 0·05 cc. Rabbit R.B.Cs. This action of the citrate in hindering lysis was often confirmed in using other serums and R.B.Cs. as illustrated below :—

TABLE 2.

Defibrinated rabbit blood	·45 cc.	Complete lysis.
·85 per cent. salt	1·50 cc.	
Sheep R.B.Cs.	·05 cc.	
Citrated rabbit blood	1·05 cc.	Nil lysis. (1·2 per cent. NaCl).
·85 per cent salt	·90 cc.	
Sheep R.B.Cs.	·05 cc.	
Defibrinated goat blood	·20 cc.	Complete lysis.
·85 per cent. salt	1·75 cc.	
Sheep R.B.Cs.	·05 cc.	
Citrated goat blood	1·25 cc.	Nearly complete lysis. (1·3 per cent. NaCl).
·85 per cent. salt	·7 cc.	
Rabbit R.B.Cs.	·05 cc.	

To determine whether the very marked difference between defibrinated and citrated bloods was due merely to the increase of tonicity consequent on the presence of 1 per cent. sodium citrate in the latter further experiments were made.

(a) On the inhibitory power of hypertonic solutions on lysis.

In a preliminary estimation it was found that the tonicity of a sheep serum was equivalent to 1 per cent. NaCl. By the addition of measured weights of dry NaCl the tonicities of three other quantities of this serum were raised to 1·2 per cent., 1·4 per cent. and 1·6 per cent. NaCl, respectively, when tested with 0·05 cc. rabbit R.B.Cs.

Serum	A	(tonicity 1·0 per cent. NaCl)	0·65 cc. gave	Complete lysis.
,,	B	(,, 1·2 ,, ,,)	1·05 ,, ,,	,,
,,	C	(,, 1·4 ,, ,,)	1·25 ,, ,,	,,
,,	D	(,, 1·6 ,, ,,)	1·65 ,, ,,	,,

So it is evident that a comparatively small increase in tonicity has a pronounced influence in the direction of hindering lysis.

(b) On the relation between the tonicities of sodium chloride and sodium citrate.

Using any R.B.Cs. as indicator, it proved that approximately three times as much sodium citrate by weight was required to prevent lysis by hypotonic solutions as was required of sodium chloride.

As long as this relation of 3 citrate to 1 chloride is borne in mind, solutions of any desired tonicity containing a mixture of the two salts in due proportions can be prepared. Thus 1 gramme sodium chloride plus 3 grammes sodium citrate dissolved in 100 cc. water is equivalent in tonicity to a 2 per cent. solution of sodium chloride. It will be noticed under experiment (*a*) that the increased tonicity of the medium did not altogether abolish lysis even up to 1·6 per cent. NaCl, but that an increased quantity of serum was required to accomplish it. In fact in the presence of 1·6 per cent. NaCl, 2·6 times as much serum was required to give complete lysis as was required in its absence.

In table 1, it may be further noticed that 1·95 cc. of citrated blood with a tonicity of 1·6 per cent. NaCl failed to lyse completely the quantity of R.B.Cs. lysed by 0·35 cc. of defibrinated blood, *i.e.*, that more than 5 parts of citrated blood failed to accomplish the same degree of lysis as 1 part of defibrinated blood.

Similarly, even when the tonicity was reduced to normal by dilution with 0·85 per cent. NaCl., 0·35 cc. citrated blood only produced slight lysis in contrast with the complete lysis produced by 0·35 cc. defibrinated blood.

Likewise, in table 2, the tonicity of the citrated bloods used does not entirely account for the total absence or hindrance of lysis there displayed.

It would appear then that citrate has some effect on blood in the way of hindering its lytic power quite apart from the tonicity of the solution.

A tonicity of 1·4 due to NaCl necessitates the use of just twice as much of the lytic serum to produce complete lysis as is required of the serum to which no NaCl has been added. In table 2 it is seen that, whereas 0·45 cc. defibrinated blood secured complete lysis, 1·05 cc. of citrated blood, more than twice as much, gave no lysis and the tonicity was equivalent to only 1·2 per cent. NaCl.

In the other example given 0·2 cc. defibrinated blood secured complete lysis, while 1·25 cc. citrated blood, more than six times as much, gave incomplete lysis with a tonicity equivalent to only 1·3 per cent. NaCl.

(c) On the effect of raising the tonicity of defibrinated blood to equal that of the citrated blood.

The tonicities of rabbit citrated blood and defibrinated blood having been determined in terms of NaCl by a preliminary experiment, the tonicity of the defibrinated blood was raised to that of the citrated by the addition of NaCl. Parallel series of tubes were then put up with the two, using goat R.B.Cs. as an indicator. The final tonicity of the two bloods was 1·63 per cent. NaCl. It was found that 0·3 cc. defibrinated blood completely dissolved 0·05 cc. goat R.B.Cs. and that 1·95 cc. of the citrated blood only partially dissolved a similar quantity.

The experiment was repeated using goat blood and rabbit R.B.Cs. The final tonicity of the defibrinated and citrated bloods was 1·6 per cent. NaCl. Complete lysis was obtained by 0·35 cc. defibrinated blood and no lysis by 1·95 cc. citrated blood.

(d) On the effect of lowering the tonicity of the citrated blood by dilution.

An experiment showed that 0·65 cc. citrated rabbit blood of a tonicity of 1·08 per cent. NaCl, and 1·95 cc. of a tonicity of 1·63 per cent. NaCl, gave an equal degree of lysis with goat R.B.Cs., proving that the lytic power of citrated blood can be to some extent, but only slightly, restored by lowering its tonicity by dilution.

Rabbit R.B.Cs. obtained from (*a*) citrated, and (*b*) defibrinated blood and washed in the usual way were lysed by fresh sheep serum in exactly similar degrees, so citrate has no permanent alterative influence on the R.B.Cs. themselves.

The inferences to be drawn from these experiments were either that the citrate precipitates some substance the presence of which in solution is essential for lysis, or that in the presence of citrate amboceptor and complement cannot combine with each other, or that amboceptor is not bound to the red cells.

The next step was to ascertain whether the addition of citrate to blood after its defibrination by whipping still inhibited its lytic power. Several experiments united in showing that the addition of 1 per cent. citrate after defibrination causes considerable diminution of lysis but not to the same degree as when the citrate is added to the whole blood. This holds good whether rabbit blood is used with sheep R.B.Cs. or goat blood with rabbit R.B.Cs.

Attempts were made to remove the citrate from citrated blood by precipitation with calcium chloride, but lysis instead of being restored was entirely abolished even when the tonicity of the solution was so far diminished by dilution as to render complete lysis possible. Other workers, however, have reported success in this experiment.

Assuming for the sake of argument that the citrate precipitates something essential to lysis from the blood no adequate explanation offers itself of the fact that the citrate has greater inhibitory power when added to the whole blood than when added to previously defibrinated blood. It can only be suggested that defibrinated blood is richer in complement on account of the mechanical disintegration of a certain proportion of leucocytes in the process of whipping, a factor of disturbance which is absent from plasma obtained by citration.

The fluid obtained after citration was always opalescent even after prolonged centrifugalisation whether the whole blood was used or whether the citrate was added after defibrination, whereas the plasma obtained by defibrination was clear. This opalescence may have been due to the precipitation of calcium citrate. It does not appear, however, if sodium citrate is added to clear serum after clotting in any proportion from 1 per cent. to 10 per cent.

Citrated blood also has a well marked agglutinating action on erythrocytes which is not found with the defibrinated blood.

The next step was to analyse citrated blood as to the activity of its lytic components.

(*a*) *Amboceptor.*—It cannot be taken for granted that lytic fluids obtained by such different methods as defibrination, clotting and citration contain equal quantities of amboceptor. In the experiments undertaken we used as a primary liquor the fluids obtained after centrifugating bloods containing varying proportions of sodium citrate, and as diluting fluids sodium citrate of similar concentrations and sodium chloride solutions isotonic with the citrate solutions.

Sensitizing mixtures were prepared as follows :—

1 per cent. citrated sheep's blood ... {
A. 5 cc. plasma plus 5 cc. of 1 per cent. sodium citrate in 0·85 per cent. NaCl.
B. 5 cc. plasma plus 5 cc. of 0·4 per cent. sodium chloride in 0·85 per cent. NaCl.

2·3 per cent. citrated sheep's blood ... {
C. 5·85 cc. plasma plus 4·15 cc. of 2·3 per cent. sodium citrate in 0·85 per cent. sodium chloride.
D. 5·85 cc. plasma plus 4·15 cc. of 0·93 per cent. sodium chloride in 0·85 per cent. NaCl.

The final solutions A, B, C and D contained equal quantities of amboceptor.

0·5 cc. of hen erythrocytes were sensitized in 10 cc. of each of these mixture for 30 minutes at 37° C and then washed so that no citrate was present in the tubes put up which could influence the action of the complement. Each tube contained 0·05 cc. of sensitized erythrocytes and the total volume was made up to 2 cc. with 0·85 per cent. NaCl. Fresh sheep serum was used as complement.

TABLE.

Complement.	A.	B.	C.	D.	E (unsensitized hen erythrocytes).
1· 0 cc.	Complete.	Complete.	Complete.	Complete.	Complete.
0·75 cc.	,,	,,	,,	,,	,,
0·5 cc.	,,	,,	,,	,,	Partial.
0·25 cc.	Partial.	Partial.	Nearly complete.	Nearly complete.	Trace.
0·125 cc.	Trace.	Trace.	Trace.	Trace.	*Nil.*
0·05 cc.	*Nil.*	*Nil.*	*Nil.*	*Nil.*	,,
0·0 cc.	,,	,,	,,	,,	,,

It appears then that the sensitization of hen erythrocytes is not interfered with by the presence of sodium citrate in concentrations of 1 per cent. or 2·3 per cent., and that it occurs to an equal extent whether the citrate solution be supplemented by similar citrate solution or by sodium chloride solution of equivalent tonicity.

A similar experiment was performed using the amboceptor from a goat which was highly immunised against sheep erythrocytes.

1 per cent. citrated goat's blood ... {
A. 1 cc. plasma plus 4 cc. of 1 per cent. sodium citrate in 0·85 per cent. NaCl.
B. 1 cc. plasma plus 4 cc. of 0·4 per cent. sodium chloride in 0·85 per cent. NaCl.

2·46 per cent. ... ... {
C. 1·08 cc. plasma plus 3·92 cc. of 2·5 per cent. sodium citrate in 0·85 per cent. NaCl.
D. 1·08 cc. plasma plus 3·92 per cent. of 1 per cent. sodium chloride in 0·85 per cent. NaCl.

4·8 per cent. ... ... {
E. 1·25 cc. plasma plus 3·75 cc. of 5 per cent. sodium citrate in 0·85 per cent. NaCl.
F. 1·25 cc. plasma plus 3·75 cc. of 2 per cent. sodium chloride in 0·85 per cent. NaCl.

7·1 per cent.	G. 1·5 cc. plasma plus 3·75 cc. of 7·5 per cent. sodium citrate in 0·85 per cent. NaCl. H. 1·5 cc. plasma plus 3·5 cc. of 3 per cent. sodium chloride in 0·85 per cent. NaCl.
10·5 per cent.	I. 2 cc. plasma plus 3 cc. of 10 per cent. sodium citrate in 0·85 per cent. NaCl. J. 2 cc. plasma plus 3 cc. of 4 per cent. sodium chloride in 0·85 per cent. NaCl.

The final solutions A to J have an equal content of amboceptor. 0·5 cc. of sheep erythrocytes were in each case sensitized in 5 cc. of mixture for 30 minutes at 37° C, then washed, so that no citrate appeared in the tubes put up.

Each tube contained 0·05 cc. erythrocytes and its total volume was made up to 2 cc. with 0·85 per cent. NaCl.

0·25 cc. fresh sheep serum sufficed in each series to produce complete lysis of the sensitized erythrocytes, so that it is clear that sensitization of the sheep erythrocytes resulted in all concentrations of sodium citrate up to 10·5 per cent.

The presence of sodium citrate in a sensitizing mixture is no bar to complete sensitization.

(*b*) *Complement—*

As a hæmolytic system human erythrocytes, sensitized in heated sheep serum, and fresh horse serum for complement were used. Horse serum by itself has little or no lytic action on human erythrocytes, whereas it is able to some extent to act as complement to sheep amboceptor.

0·5 cc. human erythrocytes A were sensitized in 5 cc. heated sheep serum for 30 minutes at 37° C and washed as usual. Another quantity B was sensitized in citrated sheep serum.

A and B were put up with fresh horse serum, citrated and uncitrated, as complement. Lysis occurred in the tubes complemented with uncitrated horse serum whether the erythrocytes had been sensitized in heated sheep serum or in citrated sheep serum. There was no lysis in any of the tubes complemented with citrated horse serum, though the tonicity was in no case higher than the equivalent of 1·2 per cent. NaCl, nor in the controls.

The citrate either as a chemical or by virtue of its added tonicity entirely inhibited complement action.

This experiment was repeated and modified in many ways, invariably with a similar result.

Summary.

(1) Raising the tonicity of a lytic serum to 1·2 per cent. NaCl by adding NaCl reduces the degree of lysis which it is able to produce.

(2) If the quantity of lytic serum in the system be increased twice or thrice complete lysis may occur even when the tonicity is equivalent to 1·6 per cent. NaCl, but if the high tonicity is due to the presence of sodium citrate even six times the needful amount of serum will not secure complete lysis.

(3) The relative tonicities of sodium chloride and sodium citrate are in the proportion of 1 to 0·3 or 0·4.

(4) If this relative tonicity of sodium chloride to sodium citrate is correct it appears as if the citrate has some effect on blood in the way of hindering lysis apart from the tonicity of the mixture.

(5) The lytic power of citrated blood can be to some extent, but only slightly, restored by dilution.

(6) The presence of sodium citrate in a mixture does not interfere with the complete sensitization of erythrocytes.

(7) The action of complement on sensitized erythrocytes is inhibited by sodium citrate. It is possible that, as in the case of the coagulation of blood, lysis of erythrocytes by amboceptor and complement is dependent on the presence in solution of calcium salts.